Allergy AND CLINICAL IMMUNOLOGY

EAACI

Allergy

ORIGINAL ARTICLE AIRWAY DISEASES

Airway and peripheral urokinase plasminogen activator receptor is elevated in asthma, and identifies a severe, nonatopic subset of patients

M. A. Portelli[1], C. Moseley[2], C. E. Stewart[1], D. S. Postma[3], P. Howarth[2,4], J. A. Warner[2], J. W. Holloway[2,5], G. H. Koppelman[6], C. Brightling[7] & I. Sayers[1]

[1]Division of Respiratory Medicine, Queen's Medical Centre, University of Nottingham, Nottingham; [2]Clinical & Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK; [3]Department of Pulmonology, Beatrix Children's Hospital, GRIAC Research Institute, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [4]NIHR Respiratory Biomedical Research Unit, Faculty of Medicine, University of Southampton; [5]Human Development & Health, Faculty of Medicine, University of Southampton, Southampton, UK; [6]Department of Paediatric Pulmonology and Paediatric Allergology, Beatrix Children's Hospital, GRIAC Research Institute, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [7]Glenfield Hospital, University of Leicester, Leicester, UK

To cite this article: Portelli MA, Moseley C, Stewart CE, Postma DS, Howarth P, Warner JA, Holloway JW, Koppelman GH, Brightling C, Sayers I. Airway and peripheral urokinase plasminogen activator receptor is elevated in asthma, and identifies a severe, nonatopic subset of patients. *Allergy* 2016; DOI: 10.1111/all.13046.

Keywords
asthma, urokinase plasminogen activator receptor, expression profile

Correspondence
Michael A. Portelli, Division of Respiratory Medicine, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH UK.
Tel.: +44 0 115 8231069
Fax: +44 0 115 31059
E-mail: michael.portelli@nottingham.ac.uk

Accepted for publication 9 September 2016

DOI:10.1111/all.13046

Edited by: Marek Sanak

Abstract

Rationale: Genetic polymorphisms in the asthma susceptibility gene, urokinase plasminogen activator receptor (uPAR/*PLAUR*) have been associated with lung function decline and uPAR blood levels in asthma subjects. Preliminary studies have identified uPAR elevation in asthma; however, a definitive study regarding which clinical features of asthma uPAR may be driving is currently lacking.
Objectives: We aimed to comprehensively determine the uPAR expression profile in asthma and control subjects utilizing bronchial biopsies and serum, and to relate uPAR expression to asthma clinical features.
Methods: uPAR levels were determined in control (n = 9) and asthmatic (n = 27) bronchial biopsies using immunohistochemistry, with a semi-quantitative score defining intensity in multiple cell types. Soluble-cleaved (sc) uPAR levels were determined in serum through ELISA in UK (cases n = 129; controls n = 39) and Dutch (cases n = 514; controls n = 96) cohorts.
Measurements and main results: In bronchial tissue, uPAR was elevated in inflammatory cells in the *lamina propria* (P = 0.0019), bronchial epithelial (P = 0.0002) and airway smooth muscle cells (P = 0.0352) of patients with asthma, with uPAR levels correlated between the cell types. No correlation with disease severity or asthma clinical features was identified. scuPAR serum levels were elevated in patients with asthma (1.5-fold; P = 0.0008), and we identified an association between high uPAR serum levels and severe, nonatopic disease.
Conclusions: This study provides novel data that elevated airway and blood uPAR is a feature of asthma and that blood uPAR is particularly related to severe, nonatopic asthma. The findings warrant further investigation and may provide a therapeutic opportunity for this refractory population.

Asthma, a chronic inflammatory airway disorder, is known to cause widespread variable airflow obstruction that is often reversible, spontaneously or with treatment, and presents with airway hyperresponsiveness (1). The urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI)-anchored membrane protein (2–4) translated from the gene *PLAUR* (4). uPAR has been associated with asthma at the biological and cellular level (5–8), with elevated uPAR

levels identified in asthma patient induced sputum (9) and bronchial epithelium (8). uPAR is a serine protease receptor and is involved in the generation of plasmin with subsequent proteolytic effects on a range of substrates; however, uPAR also has plasmin-independent functions, for example migration and proliferation, by interacting with a wide range of proteins (10). A soluble-cleaved form of the receptor (scuPAR), arising from lipolytic cleavage of the GPI anchor by factors including phospholipase D (11), also exists. scuPAR is structurally identical to the membrane-bound receptor but is able to interact with a number of cell factors, for example integrins and G protein-coupled receptors. scuPAR is thought to have functions beyond acting as a decoy receptor for the main uPAR ligands, for example uPA (12).

In a population of adult individuals, we demonstrated that higher circulating (serum) scuPAR levels were associated with asthma prevalence in 514 affected individuals when compared to 104 controls (13), suggesting a relationship between asthma and the circulating receptor. This is supported by our earlier family-based genetic association study, determining that *PLAUR* is an asthma susceptibility gene, where polymorphisms were associated with asthma, lung function, lung function decline and bronchial hyperresponsiveness in patients with asthma (7). These same *PLAUR* polymorphisms were also found to be associated with higher scuPAR levels circulating in serum in children with asthma. This suggests a link between circulating scuPAR expression and degree of asthmatic lung function, providing insight into the possible process by which scuPAR is associated with asthma.

Asthma is classified as a disease with an allergic component, with allergic asthma being a recognized endotype of the disease (14). Asthma can be defined as either atopic with triggering mechanisms involving allergens originating from, for example pollen, or nonatopic, which is not directly triggered by any known allergen, but potentially through the activation of innate receptors such as those for interleukin-33 (IL-33) and the granulocyte macrophage colony-stimulating factor (GM-CSF) (15, 16). Nonatopic asthma also generally manifests later in life and is often more severe than atopic asthma (17). Several approaches have been used to identify asthma endotypes including cluster analyses that confirm distinct atopic and nonatopic clusters (18–20).

In this study, we investigated uPAR expression profiles in lung biopsies and peripheral (serum) compartments of asthma subjects. We set out to confirm and extend previous observations carried out in a bronchial epithelial cell model (8) and serum in asthma (13), utilizing several cohorts for serum analyses and *ex vivo* bronchial biopsies. We aimed to build upon previous observations made in cell-culture models and at the genetic level by identifying whether expression in bronchial biopsies and serum are related to asthma clinical phenotypes such as lung function and allergy (atopy). We provide in-depth analysis of uPAR expression in various airway structural cells and in serum, further defining the relationship between uPAR expression and clinical features of asthma.

Materials and methods

Cohort characteristics

UK biopsy cohort

Asthmatic subjects with a history and objective evidence of asthma (21), and nonasthmatic controls were recruited from Leicester, UK. Asthma severity was defined by Global Initiative for Asthma criteria (21). Subjects underwent extensive clinical characterization including allergen skin prick tests, spirometry, methacholine bronchial challenge (PC20), sputum induction and video-assisted fiber optic bronchoscopic examination (Table S1). The study was approved by the Leicestershire Ethics Committees, and all patients gave their written informed consent.

Cohorts for serum analyses

Dutch cohort. Asthma (n = 514) and spouse control (n = 96) subjects were ascertained through an asthmatic proband and characterized using a standardized study protocol as described (13, 22). Ethical approval was obtained from the University Medical Center, Groningen Medical Ethics Committee (MEC 96/04/077 and MEC 90/09/178). Subject-based atopy was determined using the Phadiatop™ test (Phadia AB, Uppsala, Sweden), a commercially available variant of serum-specific IgE assay (23). Demographics of the Dutch cohort are shown in Table S2.

UK cohort. A cohort of 168 Caucasian individuals were recruited from the Southampton area based on an asthmatic proband (n = 129) or no history of asthma or allergic disease for the controls (n = 39) (24, 25). All individuals were between the ages of 17 and 75. Asthma severity was classified by the BTS/SIGN guidelines (26). Demographics of the UK cohort are shown in Table S3.

Immunohistochemistry (IHC) in lung biopsies from patients with asthma

Sections from control and asthmatic glycomethacrylate-embedded bronchial biopsies were stained using an anti-uPAR antibody (sc-32764; Santa Cruz Biotechnology Santa Cruz, California, United States) or an anti-mouse IgG isotype control (M7894; Sigma Gillingham, Dorset, United Kingdom). A semi-quantitative score defined epithelial expression intensity (0 = none, 1 = low, 2 = moderate, 3 = high); mean of two sections per donor, scored by a blinded observer.

Analysis of soluble protein levels

scuPAR human serum levels were determined using a Duoset® ELISA (R&D systems, Abingdon, Oxfordshire, UK). All samples were diluted 1 : 8 in 1% bovine serum albumin in phosphate-buffered saline.

Statistical analyses

Statistical analyses were carried out using SPSS (PASW) version 16.0 (IBM, North Harbour, Portsmouth, Hampshire, UK) and GraphPad PRISM version 5.03 (GraphPad Software Inc., La Jolla, California, United States). Correlation analyses were carried out on continuous variables using Spearman's correlation. Associations involving discrete variables and/or confounders for scuPAR level analyses were systematically investigated in the regression model. Previously determined confounders for scuPAR levels in serum including age in years (at time of sampling), height (in metres at time of sampling), weight (in kilograms at time of sampling) and smoking pack/year value were shown to have a statistically significant effect on scuPAR levels and were included in the final regression model as has been described previously (13). As there was *a priori* evidence of a relationship between the levels of scuPAR in serum and asthma diagnosis, we considered $P < 0.05$ significant for these analyses. In the correlation analyses, the outcome variables are not independent (e.g. FEV_1 and FEV_1/FVC), and therefore, we did not apply correction for multiple testing based on the number of tests, for example Bonferroni correction, as this would be over conservative. To provide confidence in the findings and to mitigate Type I and Type II error, we sought independent replication of findings in multiple cohorts. We therefore report *P*-values of <0.00005–0.05.

Results

uPAR is elevated in multiple airway cell types in patients with asthma

Immunohistochemical analyses of bronchial biopsies from patients with asthma ($n = 27$) and nonasthmatic controls

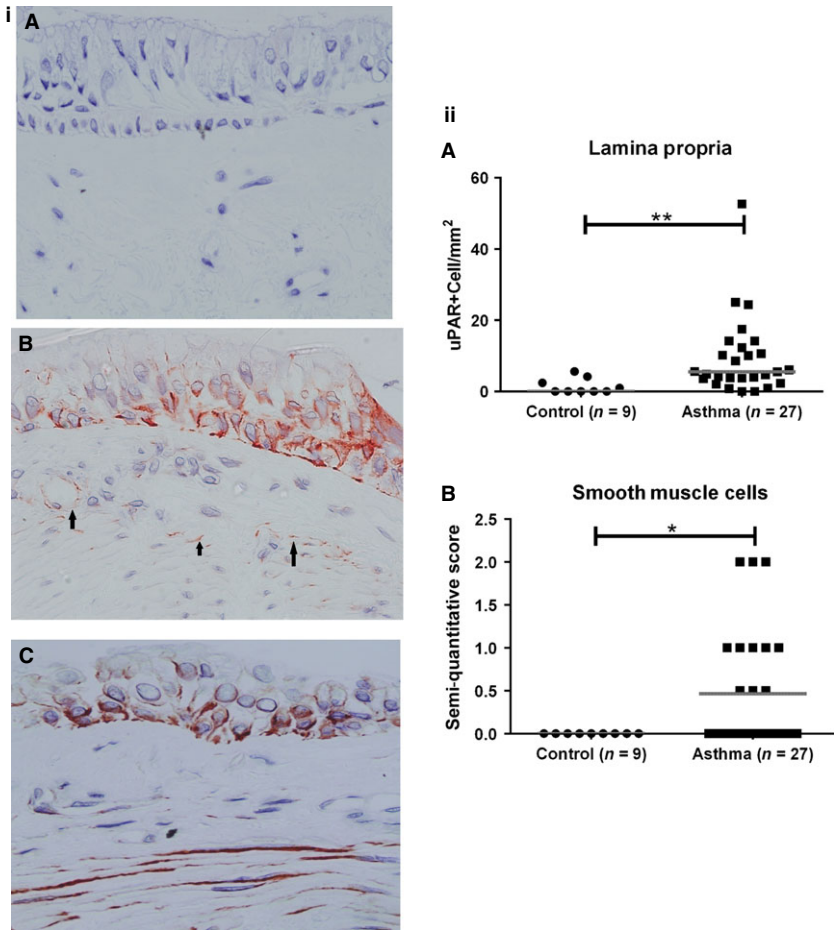

Figure 1 (i) Expression of uPAR in bronchial biopsies. (A) healthy control, (B) asthma subject revealing staining in the epithelium and cells within the *lamina propria* (highlighted by arrows) and (C) asthma subject revealing staining in the epithelium and airway smooth muscle (×400). Images are representative of a larger data set of lung biopsy slides. (ii) Median levels of uPAR are elevated in asthmatic subjects when compared to nondisease controls. Investigating the median expression levels of uPAR as defined through immunohistochemistry per number of cells within bronchial biopsies has identified an increased expression in asthma biopsies when compared to controls (A; uPAR + cells in *lamina propria* median uPAR: $P = 0.0019$). When classified into a semi-quantitative score, staining identified that this elevation of uPAR levels occurred in smooth muscle cells (B; $P = 0.0352$). *p<0.05;**p<0.01

($n = 9$) identified uPAR expression localized to inflammatory cells present in the *lamina propria*, within the cytoplasm of the epithelium and in smooth muscle cells (Fig. 1i). Expression data were grouped into a semi-quantitative score of 0 = none, 1 = low, 2 = moderate or 3 = high, as previously outlined (8). Utilizing this scoring system, we identified that uPAR expression was elevated in layers of the airway wall including the *lamina propria* and smooth muscle banding in asthmatic subjects, when compared to corresponding controls [smooth muscle cells: median 0.5 *vs* 0.0; 95% CI: 0.0–1.0 *vs* 0.0–0.0; $P = 0.0352$; uPAR + cells in *lamina propria*: median 5.536 *vs* 0.000; 95% CI: 3.73–10.64 *vs* 0.0–4.15; $P = 0.0019$ (Fig. 1ii). As previously reported, uPAR levels in the asthma epithelium were also elevated (8)]. Analyses identified clear correlations between the populations of cells staining positive for uPAR, that is if uPAR was elevated in one cell type, it was accompanied by increased staining across the tissue in the other cell types (correlation coefficient 0.2; $P < 0.05$) (Table 1). However, no significant association was identified between total uPAR levels and other clinical features of asthma including lung function and atopic status (Table 1).

Table 1 Correlation and logistic regression analyses between uPAR levels and asthma clinical phenotypes in the bronchial biopsies

Phenotype	Number	OR/r^2	*P*-value	95% CI for OR
(A) Epithelial expression in patients with asthma				
Age	27	0.002	0.820	n/a
Sex	27	0.003	0.769	n/a
Smoking pack years	12	0.030	0.593	n/a
GINA	27	0.954	0.945	0.249–3.659
Atopy	27	3.065	0.207	0.538–17.462
Baseline FEV_1 (%Pred)	27	0.021	0.475	n/a
Baseline FEV_1/FVC	27	0.005	0.729	n/a
uPAR + cells/mm^2 (*lamina propria*)	**27**	**0.208**	**0.017**	n/a
SQS ASM	**27**	**0.225**	**0.019**	n/a
(B) ASM expression in patients with asthma				
Age	24	0.024	0.468	n/a
Sex	24	0.011	0.617	n/a
Smoking pack years	11	0.119	0.285	n/a
GINA	24	3.994	0.059	0.837–14.284
Atopy	24	1.222	0.775	0.309–4.840
Baseline FEV_1 (%Pred)	24	0.121	0.096	n/a
Baseline FEV_1/FVC	24	0.029	0.426	n/a
uPAR + cells/mm^2 (*lamina propria*)	24	0.121	0.096	n/a
SQS EPI	**24**	**0.207**	**0.025**	n/a
(C) uPAR + cells/mm^2 in the *lamina propria* in patients with asthma				
Age	27	0.065	0.200	n/a
Sex	27	0.002	0.814	n/a
Smoking pack years	12	0.027	0.612	n/a
GINA	27	0.997	0.939	0.928–1.072
Atopy	27	1.084	0.123	0.978–1.202
Baseline FEV_1 (%Pred)	27	0.023	0.449	n/a
Baseline FEV_1/FVC	27	0.032	0.372	n/a
SQS EPI	**27**	0.208	**0.017**	n/a
SQS ASM	24	0.154	0.058	n/a

SQS, semi-quantitative score; EPI, epithelial cells; ASM, airway smooth muscle cells.

Spearman's correlation analyses on linear data points and logistic regression on binary values identifies a strong correlation between cellular uPAR expression between different airway cells and no association with clinical phenotypes. GINA was categorized into mild–moderate (GINA1–3) *vs* severe (GINA 4, 5) disease due to limited numbers. 95% Confidence intervals are presented for the odds ratio values.

Bold text identifies statistically significant correlations; $P<0.05$.

Elevation of serum-soluble-cleaved uPAR is a feature of asthma

To replicate our previous observation showing elevation of serum scuPAR in asthma subjects when compared to nondiseased controls (13), we utilized asthma ($n = 129$) and control subject ($n = 39$) sera from an independent UK cohort not previously used in our studies. These analyses confirmed elevation of serum scuPAR in asthmatic individuals (median scuPAR = 3056 pg/ml; 95% CI: 2706–3377) when compared to unrelated nonrespiratory disease controls (median scuPAR = 1987 pg/ml; 95% CI: 1442–2836; $P = 0.0002$) (Fig. 2A). Stratification in this cohort, based on severity as defined by the BTS/SIGN guidelines, identified that elevated serum scuPAR in asthma were primarily driven by the severe asthma subjects (BTS/SIGN ≥ 3) (Fig. 2B). Here, serum scuPAR was elevated in severe asthma ($n = 93$; median scuPAR = 3271 pg/ml; 95% CI: 2841–3568) when compared to moderate asthma ($n = 36$; median scuPAR = 2233 pg/ml; 95% CI: 1724–2838; $P < 0.01$) and controls with no history of asthma or allergic disease ($n = 39$; median scuPAR = 1987 pg/ml; 95% CI: 1442–2836; $P < 0.0001$) (Fig. 2B). No statistically significant difference was noted between controls and moderate asthma subjects (Fig. 2B).

Elevation of serum scuPAR levels is driven by a nonatopic phenotype

To investigate whether lung uPAR expression and/or circulating serum scuPAR are associated with allergic asthma, we carried out a logistic regression in the lung biopsy data set, the Dutch serum cohort that originally identified elevated scuPAR in asthma (13) and the UK serum data set based on atopic status. While no association could be identified for lung uPAR levels and atopic status (Table 1), serum scuPAR was elevated in nonatopic asthma ($n = 188$; median scuPAR = 4219 pg/ml; 95% CI: 3913–4525) when compared to the atopic asthma population using all subjects from the UK and Dutch cohorts ($n = 382$; median scuPAR = 3600 pg/ml; 95% CI: 3380–3818; $P < 0.0001$). Importantly, differential expression based on atopic status was observed independently in both cohorts (Fig. 3).

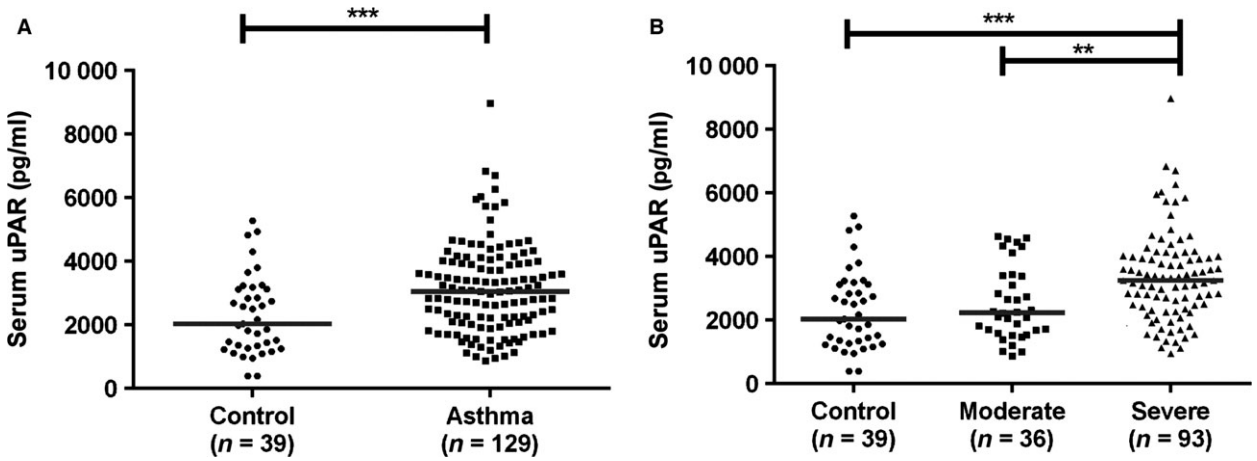

Figure 2 Elevations of serum scuPAR levels in asthma are driven by severity status in the UK cohort. (A) Measurement of serum levels of scuPAR in 129 individuals with asthma and 39 individuals with no respiratory disease from the UK cohort demonstrated that serum levels of scuPAR are elevated in asthma. (B) When stratified according to asthma severity as defined by the guidelines set out by BTS, we identify that serum scuPAR is elevated in severe asthmatics when compared to both moderate asthmatics and nonrespiratory diseased controls .**p<0.01; ***p<0.001

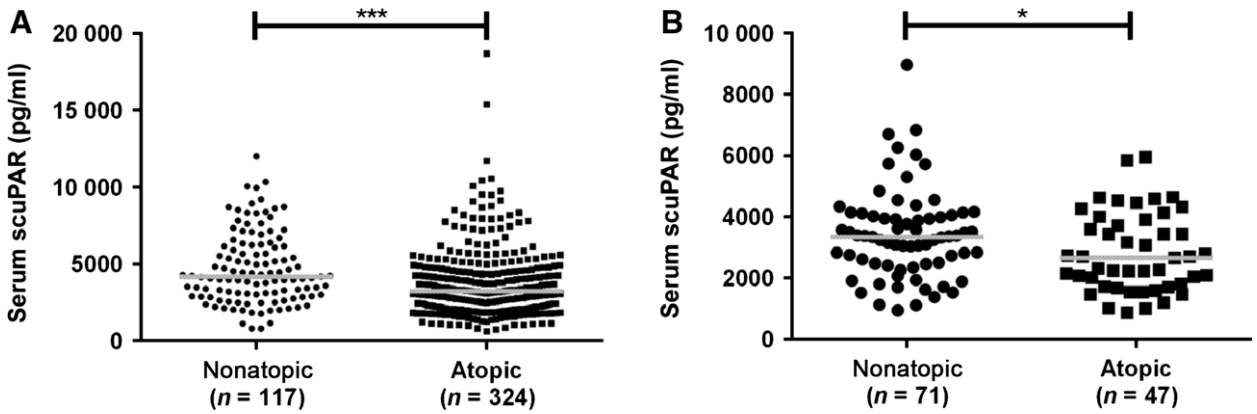

Figure 3 Serum scuPAR levels are associated with atopic status in asthma in the Dutch (A) and UK (B) data sets. Stratification of the asthmatic population according to atopic status in the UK or Dutch data sets identifies an elevated level of uPAR in nonatopic asthma *p<0.05; ***p<0.001.

Serum scuPAR levels are correlated to atopy and airway obstruction

To evaluate the correlation between scuPAR circulating levels and clinical measures in asthma, we carried out Spearman's correlation and logistic regression analyses on the UK and Dutch cohorts and the combined data set as appropriate (Table 2). We identified that circulating scuPAR levels were associated with atopic status in individual and combined cohorts using this approach (combined: OR = 1.0002; P = 0.000068) (Table 2C). In both UK and Dutch cohorts, serum levels were also positively correlated with FEV_1/FVC ratio (combined cohort: r^2 = 0.008; P = 0.035) (Table 2C). Other correlations with age (Dutch: r^2 = 0.015; P = 0.002) (Table 2A), smoking pack/years (UK: r^2 = 0.054; P = 0.009) and percentage predicted FEV_1 (UK: r^2 = 0.048; P = 0.013) (Table 2B) as well as association with steroid use (Yes/No) (Dutch: OR = 1.0002; P = 0.009) (Table 2A) were identified but did not replicate between cohorts.

Quartile analysis confirms that serum scuPAR levels are associated with atopic status

To identify whether a subpopulation drives clinical phenotype associations for serum scuPAR, we carried out a quartile analysis based on uPAR serum levels within our cohorts, separately and combined. In the Dutch data set, we identified an association with bronchodilator response measured using percentage change in FEV_1 (pre/post-BD) (mean uPAR quartile values; 19.30% > 18.99% > 16.70% > 14.80% (% change in FEV_1); P = 0.011). There was also an increase in the pre- (0.65 < 0.68 < 0.69 < 0.70; P = 0.005) and

Table 2 Correlation and logistic regression analyses between scuPAR levels in the individual UK and Dutch cohorts and combined cohorts with asthma clinical phenotypes

Phenotype	Number	OR/r^2	OR 95% CI	*P*-value
(A) Soluble uPAR in serum – Dutch Asthma Cohort				
Age	**514**	**0.0262**	**n/a**	**0.00023**
Sex	514	0.0029	n/a	0.222
Smoking pack years	320	0.0001	n/a	0.831
Atopy	**441**	**1.0002**	**1.000115–1.000306**	**0.000016**
Baseline FEV_1 (%Pred)	510	0.0045	n/a	0.132
Baseline FEV_1/FVC	**514**	**0.0132**	**n/a**	**0.009**
FEV_1 reversibility (%)	514	0.0050	n/a	0.106
Steroid use	**427**	**1.0002**	**1.000077–1.000377**	**0.009**
(B) Soluble uPAR in serum – UK Asthma Cohort				
Age	125	0.0213	n/a	0.104
Sex	125	0.0361	n/a	0.340
Smoking pack years	**53**	**0.1875**	**n/a**	**0.001**
Atopy	**118**	**1.0004**	**1.000043–1.000741**	**0.028**
Baseline FEV_1 (%Pred)	**129**	**0.0479**	**n/a**	**0.013**
Baseline FEV_1/FVC	**129**	**0.0533**	**n/a**	**0.008**
FEV_1 reversibility (%)	129	0.0199	n/a	0.111
Steroid use	76	1.000184	1.000000–1.000587	0.283
(C) Soluble uPAR in serum – Combined Asthma Cohort				
Age	**639**	**0.015**	**n/a**	**0.002**
Sex	**639**	0.004	n/a	0.593
Smoking pack years	373	0.002	n/a	0.389
Atopy	**559**	**1.0002**	**1.000089–1.000262**	**0.000068**
Baseline FEV_1 (%Pred)	643	0.004	n/a	0.104
Baseline FEV_1/FVC	**643**	**0.0083**	**n/a**	**0.035**
FEV_1 reversibility (%)	643	0.001	n/a	0.333
Steroid use	**503**	**1.000217**	**1.000164–1.000398**	**0.001**

OR, Odds ratio for logistic regression.

Correlation analyses using linear regression identified a positive relationship between the circulating uPAR and FEV_1/FVC ratio independently in both cohorts. Logistic regression identified a similar relationship between uPAR and atopic status in both cohorts and steroid use (Yes/No) in the combined and Dutch cohorts only. 95% Confidence intervals are presented for the odds ratio values.

Bold text identifies statistically significant correlations; $P<0.05$

Table 3 Interquartile analyses of the UK and Dutch cohorts (*P*-values)

	UK	Dutch	Combined
Reversibility (change FEV_1)	0.394	**0.011** (19.30% > 18.99% > 16.70% > 14.80%)	0.317
Baseline FEV_1	0.208	0.061	0.428
Post-BD FEV_1	n/a	0.256	n/a
Baseline FEV_1/FVC	0.188	**0.005** (0.65 < 0.68 < 0.69 < 0.70)	n/a
PostBronchodilator FEV_1/FVC	0.188	**0.003** (0.71 < 0.74 < 0.75 < 0.76)	0.258
Atopy	**0.044** (38.9% > 51.4% > 70.0% < 61.1%)	**<0.001** (11.0% > 18.0% > 25.2% > 35.2%)	**0.001** (17.5% > 25.3% > 36.0% > 38.1%)

BD, bronchodilator.

Analysis identifies an association with atopic status across both cohorts with some evidence for association with lung function and reversibility (change in FEV_1 postbronchodilator) in the Dutch cohort.

Bold text identifies statistically significant correlations; $P<0.05$

postbronchodilator ($0.71 < 0.74 < 0.75 < 0.76$; $P = 0.003$) FEV_1/FVC ratio across uPAR quartiles. These associations were not replicated in the UK or combined data sets (Table 3). A significant negative correlation between atopic status and the scuPAR quartiles was also observed independently and in the combined cohorts (combined nonatopic (%) across quartiles: $17.5 > 25.3 > 36.0 > 38.1$; $P < 0.0001$, Table 3).

Discussion

Accumulating evidence associates uPAR expression and function with obstructive lung disease and airway structural changes (5, 6, 8, 9, 27). In this study, we aimed to (i) determine the uPAR cellular expression profile in asthma and control subject lungs, (ii) further define the association between scuPAR levels and asthma diagnosis and (iii) investigate correlation between uPAR levels in the airways/circulation and asthma clinical features. We identified lung tissue uPAR expression in bronchial epithelial cells, airway smooth muscle cells and inflammatory cells in the *lamina propria* and demonstrate coordinated expression across these locations. Serum scuPAR was reproducibly elevated in multiple asthma cohorts. Importantly, we provide the first evidence that serum scuPAR is elevated in a more severe and in nonatopic subphenotypes of asthma. These data therefore not only confirm the relationship between the circulating uPAR and asthma, but provide novel data identifying a relationship between asthma and expression in multiple airway cell types. Critically, we highlight the potential importance of circulating scuPAR, suggesting that this molecule acts independently to the membrane-bound form and identified a new asthma phenotype 'scuPAR high' that is severe and nonatopic.

PLAUR is a known asthma susceptibility gene, with intragene polymorphisms linked to asthma diagnosis, baseline lung function and lung function decline and serum scuPAR levels (7). At the protein level, elevated uPAR has been identified in post-mortem *status asthmaticus* lungs (27), in the asthmatic bronchial epithelium and serum (8, 13). In the current study, we extend our published findings by localizing uPAR expression in bronchial tissue and identifying elevated expression in multiple structural cells in patients with asthma. Association and correlation analyses confirmed coordinated uPAR expression in lung structural and inflammatory cells. This suggests a complex relationship between different bronchial structural cells and uPAR expression, where it has been suggested that uPAR is involved in a number of airway remodelling linked processes, for example wound repair and epithelial proliferation (8, 13). These data suggest that there may be potential genetic predisposition in carriers of specific *PLAUR* genotypes, to produce elevated levels of uPAR in multiple cell types and organs; an issue we were unable to address due to lack of patient numbers and matched biopsies/serum and genetic information. In support of this concept, a recent expression quantitative trait loci study of peripheral blood cells identified that several of the asthma-associated *PLAUR* single nucleotide polymorphisms (SNPs) originally identified (7) were also determinants of uPAR mRNA levels in blood cells (28).

Based on studies identifying association between serum scuPAR levels and asthma diagnosis (13) and on genetic studies linking serum scuPAR to lung function SNPs (7), we next set out to identify whether scuPAR levels were related to asthma clinical phenotypes. In the biopsy study, we did not identify a significant association between uPAR immunohistochemical staining and any of the clinical features of asthma tested including lung function and atopy. Interestingly, a COPD study observed a significant association between small airway epithelial uPAR levels using immunohistochemistry and forced expiratory flow 25–75% (FEF_{25-75}), suggesting the anatomical location and/or the specific spirometry measure may be key (5). Similarly, our lack of association may be driven by the number of patients in our analyses and the lack of resolution of IHC to distinguish uPAR isoforms such as scuPAR which is clearly of significance in the circulation.

In the serum analyses, we confirmed previous observations that serum scuPAR is elevated in asthma. This study has now replicated that scuPAR is also associated with the asthma phenotype and therefore may also have a defining and modulatory role in asthma. Although serum scuPAR is elevated in asthma, the large degree of intersubject variability suggests that uPAR would not be useful as a marker of asthma *per se*. This expression distribution is likely due to asthma heterogeneity, that is uPAR is not an important determinant in all types of asthma. Therefore, we set out to determine whether serum scuPAR is elevated, and by inference involved in, a specific subset of patients with asthma. Asthma is a heterogeneous obstructive lung disease (1) comprised of different disease subsets (20) which have markedly different presenting symptoms and degrees of severity, some of which may have greater association with circulating scuPAR. Therefore, we hypothesized that there may be a 'uPAR driven' subset of patients with asthma. Indeed, circulating scuPAR elevations in the UK cohort appear to be driven by the severe disease (BTS/SIGN $\geq$ 3), with no elevation identified in mild–moderate asthma. Severe asthma defined using BTS/SIGN is based on asthma symptoms, and the patients have frequent severe and serious exacerbations despite taking multiple asthma medications, that is asthma control (29). We propose that the asthma phenotype identified by elevated serum scuPAR is managed to a lesser degree by current medication (BTS severe) and therefore may identify a subset of asthma where there is a therapeutic need. One potential confounder of our analyses is steroid use, with greater use of steroids being a characteristic of the severe asthma group. However, we formally tested this and show that inclusion of steroid use in the regression models did not influence this association between elevated serum scuPAR and severe asthma (data not shown). It is important to emphasize that steroid use as a dichotomous trait was included in the regression model due to the lack of data available for actual doses of corticosteroids the patients were taking. This means that while our data suggest that steroid use does not confound our statistical models and our interpretation of these data we cannot completely exclude an effect of corticosteroid use on uPAR expression.

Although disease severity may partially explain the wide distribution of serum scuPAR levels in our cohorts, the presence of a large heterogeneity of data even when separated according to asthma severity suggests that uPAR levels may be also affected by other defining features of asthma. To investigate this, we analysed the effect of atopic status on circulating scuPAR levels in the Dutch and UK cohorts, identifying that

serum scuPAR levels were elevated in nonatopic *vs* atopic asthma. As we report potential association with steroid use, significant in the Dutch cohort (Table 2A), we confirmed that no confounding of these findings by steroid use was present by running the regression model with and without this dichotomous (Yes/No) covariant. Again steroid use did not influence the statistical models used, and the association between elevated serum scuPAR in nonatopic asthma was retained (data not shown). It is important to note that the relationship between steroid use and serum uPAR levels in the Dutch cohort is inconsistent and was not observed in the UK cohort meaning that it is likely that this is related to the elevated steroid use in nonatopic patients. Analysis of uPAR expression in bronchial biopsies did not identify association with atopic status, suggesting the relationship is driven by scuPAR which in the biopsy study could not be distinguished from membrane forms. Similarly, these data may suggest that inflammatory cells (as in the blood) are one of the main sources of scuPAR in asthma. The relationship between, for example sputum scuPAR and these parameters would begin to address these differences; however, matched biopsies and serum samples were not available.

Nonatopic asthma is generally an adult onset disease, often with a more severe clinical course (30, 31). Analyses of the cohorts used in this study stratified into atopic and nonatopic asthma provide some supporting evidence for this, for example reduced FEV_1 in nonatopic asthma (Tables S2 and S3). The association of serum scuPAR to a more severe phenotype of asthma in the current study, and genetic associations between *PLAUR* SNPs and lung function in patients with asthma (FEV_1) (7) and smokers (FEV_1 and FEV_1/FVC) (32), led us to determine association between uPAR levels and lung function parameters. We confirmed serum scuPAR is negatively associated with atopic status and positively correlated with FEV_1/FVC in individual UK and Dutch cohorts and in the combined analyses, suggesting that the serum component may be a systemic marker of ongoing changes in the airway. These data suggest scuPAR as a potential marker of airway obstruction; however, the positive correlation is potentially counterintuitive, that is elevated uPAR is associated with more severe asthma but a higher FEV_1/FVC ratio suggesting it is protective for this marker of airway obstruction. Further examination of the severe asthma subjects in the UK cohort who demonstrated elevated serum scuPAR levels shows that these individuals have a reduced FVC compared to moderate asthma subjects (Table S3). However, FVC was not associated with serum scuPAR levels in this cohort (data not shown) suggesting the association with FEV_1/FVC is not driven by FVC. Also, FEV_1/FVC associations were not as reproducible as the atopy correlation, for example in the quartile analyses, the association between FEV_1/FVC and uPAR serum levels was not present in the UK cohort questioning this finding.

While we have focussed on the role of uPAR in asthma, it is important to note that this receptor may be of relevance in a range of human disorders, for example focal segmental glomerulosclerosis (FSGS) where scuPAR is elevated in disease (33, 34). Importantly, the study suggests that scuPAR was an FSGS causative factor via integrin interactions, including β[3] integrin. Although the veracity of this interaction is currently under discussion (35), there remains evidence of scuPAR as a direct modulator of disease. Two studies have identified that scuPAR has functional properties beyond acting as a decoy for uPA binding including mediating uPAR signalling as both an agonist and antagonist depending on the cellular context and promoting migration (12, 36). This may be of relevance to asthma and further investigations of the role of scuPAR and integrins in asthma are warranted.

This study has several major strengths, including the extensive analyses of both lung and systemic uPAR levels in large numbers of asthma and control subjects, to identify differential expression and the relationship of expression to clinical features of asthma. The limitations of the study include; that we did not have matched sputum, biopsy and serum samples for subjects which would have enhanced the study, as all measures were not available in all cohorts and that we could not (technically) distinguish uPAR isoforms in the airways. Similarly, limited information on the commercial ELISA used means that we cannot conclude whether both uPA bound and/or free scuPAR were measured in serum. While the subjects in our study were recruited and clinically characterized in respiratory centres, it is important to highlight that we did not collect extensive nonrespiratory comorbidity data for subjects. Therefore, although unlikely, we cannot exclude that undiagnosed comorbidities where uPAR may be involved, may have influenced our data. However, using multiple cohorts, we have mitigated this risk. It is also useful to note that the subjects contributing to the current analyses are enriched for moderate–severe asthma due to the nature of recruitment from hospital settings, therefore, our subjects may not entirely be representative of the whole population of asthma subjects.

In conclusion, this work provides novel insight into the potential role of the asthma-associated gene, *PLAUR* in asthma. Specifically, we identified that uPAR is elevated in a range of airway structural and inflammatory cells in asthma and that serum levels of scuPAR are reproducibly elevated in asthma. Importantly, we report for the first time a 'serum uPAR high' subset of asthma that is predominantly severe and nonatopic. These data highlight uPAR and in particular, the soluble form of the receptor as a potentially important target for further investigation for novel future approaches to treat asthma, particularly in severe refractory asthma where new medicines are needed.

Author contributions

Experimental work: MAP, CMM, CES. Conception and design of the study: MAP, IS. Analysis and interpretation: MAP, CMM, CES, DSP, PH, JAW, JWH, GHK, CEB, IS. Drafting the manuscript for important intellectual content: MAP, CMM, CES, DSP, PH, JAW, JWH, GHK, CEB, IS.

Financial disclosure

1 STEPS scholarship part financed by the European Union – European Social Fund (ESF) under Operational

Programme II – Cohesion Policy 2007–2013, 'Empowering People for More Jobs and a Better Quality of Life'
2 Asthma UK, Grant 08/017.
3 The study is also part funded by the National Institute for Health Research Leicester Biomedical Research Unit, UK. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or Department of Health.

Conflicts of interest

Dr. Portelli, Dr. Sayers, Dr. Stewart, Dr. Holloway and Dr. Warner have nothing to disclose. Prof. Brightling has received research grants and consultancy via his Institution from GSK, AZ, Novartis, Chiesi, BI and Roche, outside the submitted work. Dr. Postma reports grants to the university from Astra Zeneca, Boehringer Ingelheim, GSK, Takeda, TEVA, outside the submitted work. Dr. Koppelman reports grants from the Netherlands Lung Foundation, during the conduct of the study; grants from Ubbo Emmius Foundation, grants from TEVA Pharmaceuticals, grants from Stichting Astma Bestrijding, outside the submitted work.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical and sputum characteristics of lung biopsy groups.

Table S2. Demographic information for the Dutch cohort used for serum analyses.

Table S3. Demographics information for the UK cohort used for serum analyses.

References

1. Shaw D, Portelli MA, Sayers I. Asthma. In: Padmanabhan S, editor. *Handbook of pharmacogenomics and stratified medicine*. London: Academic Press, 2014: 617–652.
2. Plesner T, Ploug M, Ellis V, Ronne E, Hoyer-Hansen G, Wittrup M et al. The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. *Blood* 1994;**83**:808–815.
3. Ploug M, Behrendt N, Lober D, Dano K. Protein structure and membrane anchorage of the cellular receptor for urokinase-type plasminogen activator. *Semin Thromb Hemost* 1991;**17**:183–193.
4. Ploug M, Ronne E, Behrendt N, Jensen AL, Blasi F, Dano K. Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. *J Biol Chem* 1991;**266**:1926–1933.
5. Wang IM, Stepaniants S, Boie Y, Mortimer JR, Kennedy B, Elliott M et al. Gene expression profiling in patients with chronic obstructive pulmonary disease and lung cancer. *Am J Respir Crit Care Med* 2008;**177**:402–411.
6. Wang Q, Wang Y, Zhang Y, Zhang Y, Xiao W. The role of uPAR in epithelial-mesenchymal transition in small airway epithelium of patients with chronic obstructive pulmonary disease. *Respir Res* 2013;**14**:67.
7. Barton SJ, Koppelman GH, Vonk JM, Browning CA, Nolte IM, Stewart CE et al. PLAUR polymorphisms are associated with asthma, PLAUR levels, and lung function decline. *J Allergy Clin Immunol* 2009;**123**:1391–1400.
8. Stewart CE, Nijmeh HS, Brightling CE, Sayers I. uPAR regulates bronchial epithelial repair in vitro and is elevated in asthmatic epithelium. *Thorax* 2012;**67**:477–487.
9. Xiao W, Hsu YP, Ishizaka A, Kirikae T, Moss RB. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. *Chest* 2005;**128**:2316–2326.
10. Portelli MA, Stewart CE, Hall IP, Brightling CE, Sayers I. Cigarette smoke induces uPAR in vivo and isoforms selectively contribute to bronchial epithelial phenotype. *Am J Respir Cell Mol Biol* 2015;**53**:174–183.
11. Thuno M, Macho B, Eugen-Olsen J. suPAR: the molecular crystal ball. *Dis Markers* 2009;**27**:157–172.
12. Jo M, Thomas KS, Wu L, Gonias SL. Soluble urokinase-type plasminogen activator receptor inhibits cancer cell growth and invasion by direct urokinase-independent effects on cell signaling. *J Biol Chem* 2003;**278**:46692–46698.
13. Portelli MA, Siedlinski M, Stewart CE, Postma DS, Nieuwenhuis MA, Vonk JM et al. Genome-wide protein QTL mapping identifies human plasma kallikrein as a post-translational regulator of serum uPAR levels. *FASEB J* 2014;**28**:923–934.
14. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol* 2011;**127**:355–360.
15. Barnes PJDJ, Rennard S, Thomson N, editors. *Asthma and COPD*. London: Academic Press; 2002.
16. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN et al. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med* 2012;**209**:1505–1517.
17. Rochat T. [Is intrinsic or nonatopic asthma a particular disease?] *Rev Med Suisse* 2005;**1**:2640–2643.
18. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;**181**:315–323.
19. Siroux V, Basagana X, Boudier A, Pin I, Garcia-Aymerich J, Vesin A et al. Identifying adult asthma phenotypes using a clustering approach. *Eur Respir J* 2011;**38**:310–317.
20. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008;**178**:218–224.
21. O'Byrne PM. Global guidelines for asthma management: summary of the current status and future challenges. *Pol Arch Med Wewn* 2010;**120**:511–517.
22. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 2009;**180**:929–935.
23. Merrett J, Merrett TG. Phadiatop–a novel IgE antibody screening test. *Clin Allergy* 1987;**17**:409–416.
24. Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W et al. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005;**60**:1012–1018.
25. Wan YI, Shrine NR, Soler Artigas M, Wain LV, Blakey JD, Moffatt MF et al. Genome-wide association study to identify genetic

determinants of severe asthma. *Thorax* 2012;**67**:762–768.
26. O'Byrne P, Chair. *Global strategy for asthma management and prevention. WHO/NHLBI workshop report*. MRC Vision Inc., New Jersey: Lung and Blood Institute: National Institutes of Health, National Heart; 1995.
27. Chu EK, Cheng J, Foley JS, Mecham BH, Owen CA, Haley KJ et al. Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2006;**35**:628–638.
28. Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. *Nat Genet* 2013;**45**:1238–1243.
29. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;**43**:343–373.
30. Rackemann FM. Intrinsic asthma. *Bull N Y Acad Med* 1947;**23**:302–306.
31. Humbert M, Menz G, Ying S, Corrigan CJ, Robinson DS, Durham SR et al. The immunopathology of extrinsic (atopic) and intrinsic (nonatopic) asthma: more similarities than differences. *Immunol Today* 1999;**20**:528–533.
32. Stewart CE, Hall IP, Parker SG, Moffat MF, Wardlaw AJ, Connolly MJ et al. PLAUR polymorphisms and lung function in UK smokers. *BMC Med Genet* 2009;**10**:112.
33. Wei C, El Hindi S, Li J, Fornoni A, Goes N, Sageshima J et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat Med* 2011;**17**:952–960.
34. Huang J, Liu G, Zhang YM, Cui Z, Wang F, Liu XJ et al. Urinary soluble urokinase receptor levels are elevated and pathogenic in patients with primary focal segmental glomerulosclerosis. *BMC Med* 2014;**12**:81.
35. Maas RJ, Deegens JK, Wetzels JF. Serum suPAR in patients with FSGS: trash or treasure? *Pediatr Nephrol* 2013;**28**:1041–1048.
36. Gilder AS, Jones KA, Hu J, Wang L, Chen CC, Carter BS et al. Soluble urokinase receptor is released selectively by glioblastoma cells that express epidermal growth factor receptor variant III and promotes tumor cell migration and invasion. *J Biol Chem* 2015;**290**:14798–14809.